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Goals and Accomplishments of the Corvallis Risk Assessment Biotechnology Program

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GOALS AND ACCOMPLISHMENTS OF THE CORVALLIS RISK ASSESSMENT BIOTECHNOLOGY PROGRAM

A presentation given at the "Risk Assessment in Agricultural Biotechnology" held at University of California, Davis, July 31-August 4, 1988

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ABSTRACT

The goal of the Terrestrial Microbial Ecology/Biotechnology Program is to develop methods to evaluate potential impacts of genetically engineered microbes (GEMs), and microbial pest control agents (MPCAs), on terrestrial ecosystems. In the last few years, biotechnology companies have constructed genetically engineered viruses, bacteria, and fungi that will function following their purposeful release into the environment. This technology has brought a renewed interest in microbial ecology and a need to define and better understand the nature of exposures and possible risks of releasing these novel microbes. Our efforts have dealt largely with the development of complex microcosms to study the kinetics of conjugal gene transfer, survival, and dispersal of various bacterial genera containing recombinant DNA. We have found that insects can serve as reservoirs of recombinant organisms and as vectors causing contamination of plants and soil. Evaluation of our data on aerosol dispersal of recombinant bacteria spray released in California revealed that simple monitoring devices can be used to document the extent of bacterial dispersal. Ultimately, data from laboratory, microcosm, and field evaluations will be integrated into mathematical models that will help predict the ramifications of releasing recombinant microbes into the environment.

INTRODUCTION

The progress that biotechnology companies have made in the last few years in the construction of genetically engineered microbes (GEMs) has been paralleled by an increased interest in biotechnology by the public and by Congress. This has led to increased activity among regulatory agencies, including the U.S. Environmental Protection Agency (EPA). The EPA is authorized to regulate GEMs for environmental release under the Federal Insecticide, Fungicide, and Rodenticide Act, (FIFRA) and the Toxic Substance Control Act (TSCA).

The internal structure of the EPA is such that the research efforts are conducted by the Office of Research and Development (ORD) in support of organizationally-separate regulatory offices. The ORD is responsible for developing and evaluating protocols and methods that can be used to assess the impact on both the environment and human health of industrial products intended for release (Levin et al. 1987).

EPA BIOTECHNOLOGY RESEARCH PROGRAMS

ORD's formal involvement in biotechnology risk assessment research started with a series of workshops in 1978. These workshops were intended to target research areas where the agency should concentrate on in its mission of regulating the environmental biotechnology industry.

In 1985, ORD involvement was expanded with the establishment of a biotechnology risk assessment research program. One part of the program is being conducted at the Environmental Research Laboratory in Gulf Breeze, Florida, which is devoted to aquatic ecosystems. The other part in place at the Environmental Research Laboratory in Corvallis, Oregon (ERL-C), is responsible for terrestrial ecosystem risk assessment research. Supporting the Corvallis program are 23 scientists, technicians, postdoctoral fellows, and students representing both on-site contract personnel and full-time EPA personnel. This laboratory also manages an extensive ongoing extramural research effort in conjunction with about two dozen universities.

ERL-C PROGRAM

The objective of the program in Corvallis is to develop test methods for identifying and predicting the consequences of releasing GEMS into terrestrial ecosystems. The program involves work on fungi, viruses, and bacteria. This presentation will concentrate on the bacterial aspects of this program.

An integral part of this research focuses on microbial pest control agents (MPCAs). This work is designed to test existing or develop protocols for the Agency to use in examining the effects of both recombinant and nonrecombinant microbial pesticides on nontarget beneficial arthropods and avian species. The ongoing arthropod research in Corvallis involves establishing procedures for the cultivation of predatory Neuroptera, mites, Coleoptera, and parasitic Hymenoptera. These beneficial species are then exposed, under highly controlled environmental conditions, to suitable candidate MPCAs representing viral, bacterial, fungal, and protozoan agents. Lethal and sublethal effects are documented and the possible effects of environmental changes on MPCA virulence are noted. The avian species being used are the bobwhite quail (Colinus virginianus) and the mallard duck (Anasplaty rhynchos,) both standard wildlife toxicology models for avian species. Researchers in this area are developing protocols for assessing the effects of environmental exposures to the same major groups of microbial pest control agents.

The experimental ecological analysis research on GEMs is concentrated on improving detection and enumeration methods, evaluation of factors that influence survival and transport, and genetic exchange. The Corvallis program also has responsibility for field design of environmental release protocols and for the development of methods of evaluating the effects of GEMs on ecological processes. Research is ongoing in all these areas. In this presentation we discuss three projects involving fate/survival, identification/enumeration and transport to show the background of the program, its present status, and our plans for its evolution.

GEM SURVIVAL

Understanding GEM survival is fundamental to the entire scope of problems in environmental biotechnology. If the microbes do not survive in the environment, it is unlikely that they will be able to do the job they were intended to do or to significantly influence the environment into which they are released.

The ERL-C EPA biotechnology program has been using microcosms for evaluating the survival and fate of GEMs. Our working definition of a microcosm is "a scientific test system that attempts to simulate a portion of the natural environment." The microcosm is not designed to replace the natural environment for environmental research. The microcosm is a tool that is used

to bridge the gap between highly artificial laboratory environments and the natural environment. It is also a convenient means of obtaining an approximation of the effect on the environment produced by a GEM which is maintained in a contained system.

Two experimental microcosm systems have been developed at ERL-C. Both utilize 1 m^3 enclosed plexiglass chambers originally developed for chemical effects research (Gile and Gillett, 1981). Air temperature, wind speed, photoperiod, and relative humidity are monitored and controlled in the chambers. Soil and plants are maintained either in double plastic pots (up to 20 per microcosm) or in plastic trays. In the double pot system, the lower pot feeds a plant nutrient solution to the upper soil/plant pot via polyester cord wicks threaded through the bottom of the upper pot. This arrangement is used to study the survival and genetic exchange of bacteria growing in the rhizosphere and was tested using barley (Hardeum vularge, cultivar Advance) and radish plants (Raphanus sativus, cultivar Cherry Belle). The trays are used for studying the survival and genetic exchange among GEMs present on the phyllosphere.

Monitoring of environmental conditions within the microcosm is accomplished using a personal computer and a Campbell 21 X micrologger to measure and store data on leaf wetness, soil moisture, soil and air temper- ature, photoperiod, relative humidity; and a personal computer.

Environmental Perturbations

Experiments have been conducted to evaluate the specific effects of environmental perturbations on GEM survival. For example, Figures 1 and 2 show the effects of high and low relative humidity and soil moisture on the survival of Enterobacter cloacae (pBR322) in soil and in the leaf phyllosphere. This strain was isolated from the foregut of a variegated cutworm (Peridroma saucia). The survival of this organism was monitored over a 12-day period. Figure 1 illustrates that the microbe exhibited similar survival characteristics in the phyllosphere under conditions of low and high relative humidity. The E. cloacae survived better in soil, but it required high soil moisture and relative humidity. The E. cloacae strain died out very rapidly from initial populations in the soil under low humidity, although it was not entirely eliminated after 12 days.

More involved GEM survival and transport studies are being conducted by adding variegated cutworms to the soil/plant microcosm. A means of propagating these insects was developed in the laboratory and large numbers are available for experimental inoculations with GEMs. Research is being conducted to determine if these insects can serve as vectors and reservoirs for GEM transmission from an inoculated environment to an uninoculated environment. The experimental protocols simulate the spraying of a GEM into a field in which insects are present or to which they will migrate after the spray. Insects consuming leaf matter ingest the GEM that has been applied.

Erwinia herbicola pBR322 was incubated for 18 hr at 30° C using a rotary shaking incubator at 200 rpm, in LB broth (Maniatus, 1982) containing the appropriate antibiotics. The culture was washed in 1.2 mM phosphate buffer (pH 7.2) and resuspended at a population of 1 X 10^7 cfu/ml. Bean plants in tray microcosms were sprayed until run-off and transferred into microcosm chambers. Insects were added and allowed to feed on inoculated plants for three days and were then transferred into uninoculated microcosms.

Results (figure 3) show that after three days, the initial population on E. herbicola were about 5 X 10^4/g, but decreased by almost 1,00-fold within 24 hrs of transfer into uninoculated microcosms. In the same time period, the soil population increased from undetectable levels to about 5 X 10^4/g, then began a slow decline. The E. herbicola population on the leaves began to increase after 24 hrs, reaching a peak population at day two after transfer. The increase on the leaves coincided with an increase in the foregut of the insects, as did a decline from days two to four.

The initial decrease in the foregut was probably due to the absence of the organisms on the leaves of the second microcosm. As the insects fed on the uninoculated leaves, they eliminated the organism from their digestive tract. The rapid increase of the E. herbicola in soil was possibly the result of the insects migrating from the leaves to the soil during light periods, allowing inoculation of the organism from the surface of the insects, and from deposition of frass pellets. The same method was probably responsible for inoculation of the leaves.

Seven days after introduction into the new microcosm, the insects had devoured all leaf matter, and were removed. However, soil populations were monitored for a total of 20 days, during which time a low but stable population of E. herbicolapBR322 was established.

Detection and Transport of GEMs in the Environment

Detection of GEMs released into the environment is another area fundamental to the question of environmental biotechnology. If GEMs cannot be isolated and identified following release into the natural environment, it will be impossible to evaluate or establish correlations between possible environmental effects and the release of the GEM.

Currently, the most common method of detection and enumeration of GEMs has been to rely upon resistance to antibiotics and/or heavy metals. These techniques are established as sensitive and reliable. However, the use of selective media subjects the GEMs to physiological stress that they may be unable to overcome. They may thus fail to develop colonies on agar plates. In addition, microorganisms have been known to lose resistance markers in the absence of selective pressure. Members of the indigenous

microflora may also exhibit the same resistance markers, thus limiting sensitivity and accuracy in GEM identification and enumeration. Other methods of isolation and enumeration currently under investigation include the use of fluorescent antibodies, bioluminescence, and colorimetric marker assays, such as the lac ZY genes (McCormick 1986).

A colorimetric assay currently under investigation at ERL-C is the use of the xylE gene of the Tol pathway. The Tol pathway (Figure 4) enables bacteria to degrade toluene to acetaldehyde and pyruvate (Inouye et al.1981). The xylE gene encodes the enzyme 2,3, catechol dioxygenase, which converts catechol to the yellow compound 2-hydroxysemimuconic aldehyde. In the absence of the remainder of the pathway, this product accumulates and imparts a yellow color. Colonies carrying the xylE gene turn yellow upon contact with catechol.

The strategy used in the initial cloning was developed in conjunction with Dr. Ron Olson of the University of Michigan. The lower portion of the TOL pathway (Figure 4) was inserted into a cloning vector pRO1614 to produce pRO1733. The 2kb fragment containing the xylE gene was isolated by deletion of the downstream portion of the pathway from pRO1733 and was designated as pRO1940. The 2kb fragment was then subcloned into pBR322 to form pMW144. This construct was transformed into Escherichia coli and E. herbicola.

To determine the effectiveness of this technique, target microorganisms were released and tracked in three different microcosm environments: (1) the phyllosphere, (2) soil contained in a tray microcosm, and (3) primary sewage influent. Target microbes were recovered on nonselective LB agar, on LB containing ampicillin, (Amp) and ampicillin and nalidixic acid (AmpNal).

Results indicated virtually no difference in the number of target microbes recovered on selective and nonselective media, until GEM populations dropped to about 1 X 10^3/g or ml in soil and wastewater, respectively. In the phyllosphere, detection limits of the GEM averaged about 10/g on nonselective LB, due to the lack of indigenous microbes. On nonselective media, sensitivity was limited in samples from soil and wastewater by the large number of indigenous microbes present in both of these environments. However, in the presence of both Amp and AmpNal, sensitivity of GEM detection was increased to about 10/g or ml. The effectiveness of this technique thus appears to be a function of the number of indigenous microorganisms that may be present in the environment in which the GEM is released. The fact that this technique was highly sensitive in the presence of Amp alone indicates that it may allow the use of antibiotics other than rifampicin and nalidixic acid in soil.

The release of the ice-minus bacteria in the spring of 1987 by Dr. S. Lindow (UC Berkeley) at Tulelake, CA and by Dr. J. Lindemann (AGS, Oakland, CA.) at Brentwood, CA, gave the agency an

opportunity to determine the extent to which microorganisms sprayed onto a site drift to adjacent areas. The objectives of the ORD sponsored aerobiology research project were: (1) to design and test a sampling procedure to determine the drift of recombinant Pseudomonas species during aerosol application and on subsequent days, (2) to determine the distribution of GEMs on and off the plot during the spray and post-spray periods, and (3) to provide recommendations for evaluating the fate and transport of recombinant organisms for future field releases (Seidler and Hern 1988).

A number of different air sampling devices were used and compared to determine recovery efficiencies. The mechanical samplers included all glass-impingers (AGIs), designed to measure total population; Andersen six-stage cascade samplers, designed to measure droplet size; and Reynier slit samplers, designed to measure populations/unit time. In addition, 150 mm petri dishes were used as gravity settling plates and trays of plants were placed around the plots as centinal plants as additional means of sampling. Meteorological measurements of wind speed and direction, relative humidity, radiation, and temperature were collected at canopy to 30 m. Aerosol samples were collected during and up to 30 minutes after the sprays, and on days 1,2, and 4 following the spray.

Target microbes were detected on gravity plates in decreasing numbers up to 15 m from the site of application at Brentwood and 30 m at Tulelake. As expected, populations were greatest inside the plot containing the sprayed vegetation, and declined rapidly as the spray plume drifted further from the source. In addition, a definite directional gradient was evident indicating that although wind conditions during the spray were calm, only a slight breeze was required to move target microbes into the buffer zone.

Significantly higher numbers of organisms were detected using the AGI samplers, which collected 15 L of air per minute. Counts ranging from 50 to over 300 recombinant Pseudomonas/m^3 were detected up to 15 m from the site of application.

GEMs were detected further from the edge of vegetation during the Tulelake release than during the Brentwood release. This can probably be attributed to the larger area sprayed at Tulelake compared to that at Brentwood. A larger plot configuration would spread the released GEMs over a larger surface area and greater drift might be expected. The source strength at the Tulelake release was also slightly greater than it was at Brentwood (4×10^3 vs 1×10^3 CFU/petri dish, respectively, at the edge of the vegetation). The droplet sizes detected at Tulelake, as measured by Andersen samplers were found to be larger than those at Brentwood. All these factors, alone or in combination, could explain the greater average drift recorded at Tulelake.

SUMMARY

In summary, the primary goal of the biotechnology program in Corvallis is to develop methods suitable for assessing the potential impact resulting from the release of GEMs into the environment. The research areas being investigated focus on methods of examining environmental effects, GEM survival, detection and transport, and genetic exchange.

Accomplishments of the program in fulfilling these goals include development of laboratory methods and microcosms to measure survival and genetic exchange, development and testing of a colorimetric assay for use in detecting, tracking and enumerating GEMs from environmental samples, and design and implementation of air sampling systems for monitoring aerosol release of GEMs in the field.

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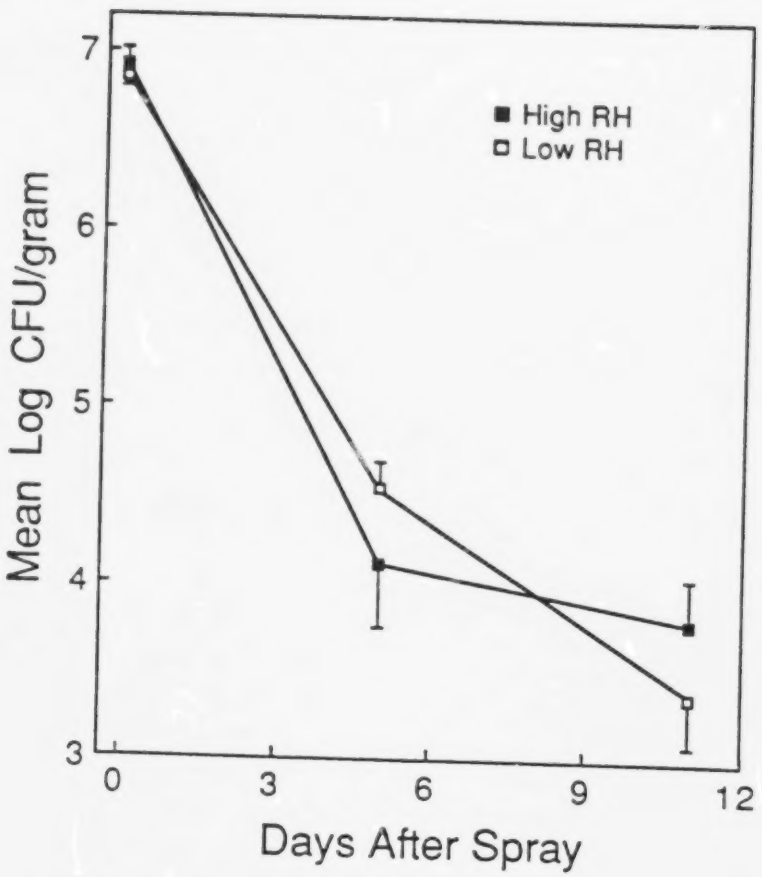
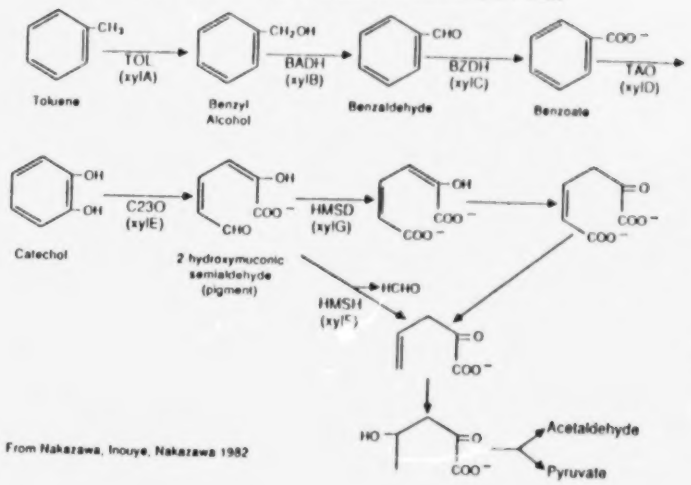
Figure 2. Survival of Enterobacter cloacae(pBR322) in soil during high and low soil moisture and relative humidity. The means of the log CFU's from 6 samples are plotted with standard error bars.

Figure 3. Appearance of Erwinia herbicola(pBr322) in an unsprayed microcosm after introduction of cutworms fed radishes inoculated with E. herbicola(pBR322). The means of the log CFU's from 3 samples are plotted with standard error bars.

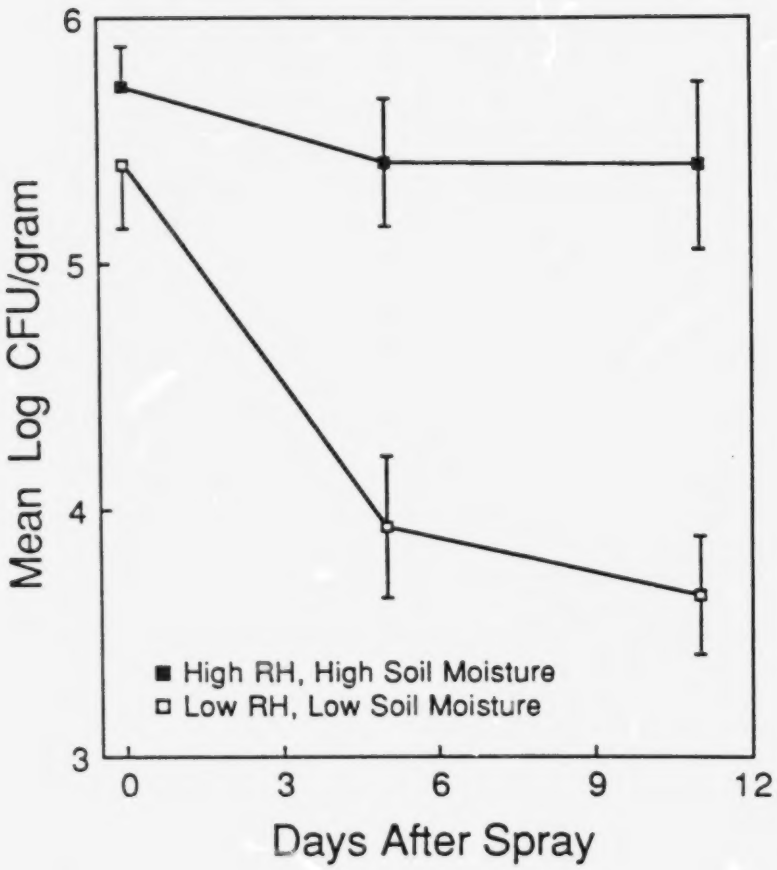
Figure 4. The Tol pathway for the degradation of toluene and benzoate in Pseudomonas aeruginosa.

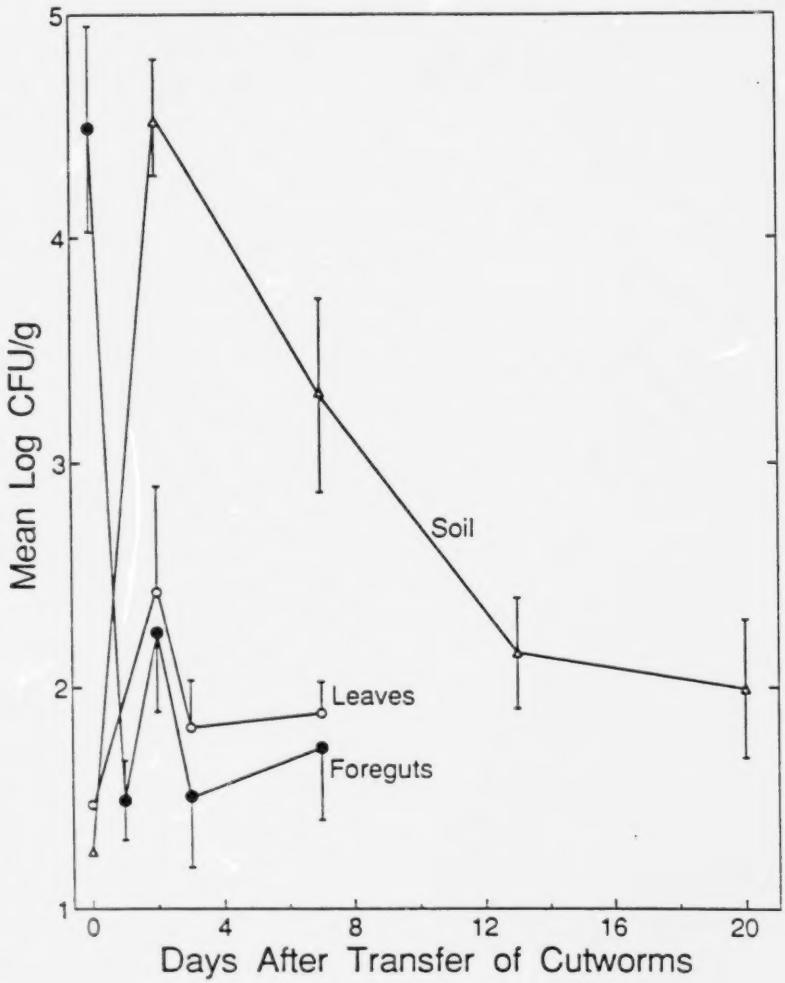

Mean Log CFU/gram
7
6
5
4
3
0
3
6
9
12
Days After Spray
High RH
Low RH

THE DEGRADATIVE PATHWAY ENCODED BY TOL



From Nakazawa, Inouye, Nakazawa 1982


6
5
4
3
Mean Log CFU/gram
0
3
6
9
12
Days After Spray
High RH, High Soil Moisture
Low RH, Low Soil Moisture


Mean Log CFU/g
5
4
3
2
1
Soil
Leaves
Foreguts
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4
8
12
16
20
Days After Transfer of Cutworms